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				Poromorto, Dewi Indriyani Roslim	School of Universitas Sebelas Maret,
34.	14:50-15:00	177	Genetic Relationships of Yardlong Bean (*Vigna unguiculata* Ssp. *sesquipedalis*) and Their F1 Progenies Based on RAPD Markers	Syaiful Anwar, Karno Karno, Florentina Kusmiyati*	Diponegoro University, Indonesia
35.	15:00-15:10	106	Composition of Planting Media and Biological Agents to Improve Physical, Chemical Properties of Soil and Lettuce (*Lactuca sativa* L.) Production	Kharisun, Fadillah, Mujiono, and Suciati	Agriculture Faculty, The University of Jenderal Soedirman

Genetic relationships of yardlong bean (*Vigna unguiculata* ssp. *sesquipedalis*) and their F1 progenies based on RAPD markers

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Abstract. Yardlong bean is a popular vegetable with moderately high nutritive value and is versatile in use. The genetic relationships of yardlong bean should be explored for long term success in its breeding programs. The aim of investigation was to evaluate genetic relationships among yardlong bean parentals and their F1 progenies, by using DNA marker analysis. RAPD was performed on DNA samples of three yardlong bean parentals and their progenies: 5 F1 from diallel crosses. Five primers generated 16 bands, ranging in size from 280 to 1160 bp, with the average number of bands per primer of 3.20. Cluster analyses using UPGMA procedure were performed using MVSP 3.1 software and produced a dendrogram with two clusters. Jaccard similarity matrices among yardlong bean parentals and their F1 progenies varied between 0.40 – 0.73. The results indicate that use of RAPD markers on DNA samples can be fast way for differentiation of yardlong bean parentals and their F1 progenies, as well as for evaluation of their genetic relationships.

Keywords: crosses, genetic relationships, yardlong bean breeding

1. Introduction

The leguminosae genus *Vigna* is comprising about 100 species mainly found in Africa and Asia: include the cowpea (*Vigna unguiculata* L. Walp) and yardlong bean (*Vigna unguiculata* L. Walp ssp. *unguiculata* cv. –gr. *sesquipedalis*) which are differ phenotypically as result of divergent selection during the crop evolution [1]. Yardlong bean is widely grown in West Africa, South China, and Southeast Asia [2]. In Indonesia, yardlong bean is known as *kacang panjang* which produces delicious crisp and tender pods that are consumed both fresh and cooked [3], and it is containing high digestible protein (23.52 – 26.27 %) along with vitamin A, thiamin, riboflavin, calcium, phosphorus, sodium, potassium, magnesium, vitamin C, iron, zinc, manganese and cobalt [4]. Annual production at 461239 tons covering about 84798 ha (average 5.5 tons ha^{-1}) [5]. Moreover, breeder try to increase the yields because of yardlong bean economical important to meet consumers high demand [6]. Genetic diversity has been considered as an important factor and pre-requisite for successful breeding program [7].

Yardlong bean has wide polymorphism in respect of flower color, fruit color, fruit size and weight, pod size, color, texture, etc. [8]. Many scientific researches looking into the nature of yardlong bean genetic diversity, as well as in the uses of genetic data in its breeding stategies [9 - 12]. Over the years, the methods for assessing genetic diversity have extended from analysis of discrete morphological to

biochemical and molecular characters, and then several PCR-based marker were developed [13]. Therefore, breeder consider marker assisted selection (MAS) a useful way in plant beeding program, because: the direct phenotypic selection is more expensive or time-consuming, the expression of the genes requires specific conditions, the heritability is low and the phenotypic selection is less efficient, and multiple genes for the same character are cumulatively under selection [14]. Genetic diversity within and among yardlong bean have been assessed by using various types of DNA markers: AFLP [15], RAPD [8], SSR [16], SNP [17], ISSR and SCoT [18].

Among DNA markers, RAPD is generally considered a fast, informative and inexpensive marker, which despite dominance and low reproducibility, allow analysis of the polymorphism in many individuals with good coverage of the entire genome [19]. RAPD have been used to determine the genetic relationships among germplasm of yardlong bean would be very useful to preservation, as well as for efficient utilization of the germplasms, especially for breeding purposes. Previously, we have assessed parental and their F1 progenies based on their morphological markers. Since the information about germplasm diversity and relationships within and among breeding material is important for any yardlong bean breeding program, the aim of this research was to evaluate genetic relationships among yardlong bean parental and their F1 progenies, by using RAPD markers. The information obtained represent the initial evaluation of the potential usefulness of RAPD markers as an inexpensive, quick and efficient tool for diversity screening, and possible application of MAS in yardlong bean breeding programs.

2. Materials and Methods

The yardlong bean cultivars: Fagiola Ungu (FG), Aura Hijau (AH) and Super Putih (SP), were chosen because of their divergent morphological characters (Table 1), and used as parents in a complete diallel with reciprocals (FG x AH, FG x SP, AH x FG, AH x SP, SP x FG, SP x AH). The method proposed by [20], which consisted of mechanical emasculation of the female parent using forceps on flower buds one day before flowering followed by crosssed pollination using ripe pollen from open flowers of the male parents, was used to perform the crosses and 5 F1 hybrids were obtained (AH x FG was not included because no seed was available). The populations consisting of three parents and 5 F1, totaling 8 treatments were assessed in the greenhouse at the Laboratory of Physiology and Crop Breeding, Diponegoro University, Semarang in early 2018. The experimental design was a randomized complete design with three replications. Four seeds were sown in 25 cm^3 pot containing substrate. They were thinned seven days after emergence, and two plants were left in each pot. Each pot with two plants made up a experimental unit. Crop management was as recommended for the yardlong bean crop [21].

Table 1. Important morphological characters and sources of three yardlong bean cultivars

Name of cultivar	Important morphological caracters				Sources
	Pod color	No. of pods per plant	Pod length (cm)	No. of seeds per pod	
Fagiola Ungu	Purple	5.78	32.81	11.65	Benih Dramaga - IPB
Aura Hijau	Green	2.67	47.98	12.58	CV. Aura Seed Indonesia
Super Putih	White	4.89	36.63	10.07	PT. BISI International, Tbk.

Yardlong bean DNA was extracted from 1 g healthy leaves, harvested from plants grown in the greenhouse, using the Plant Genomic DNA Kit (DP305; Tiangen Biotech-Beijing Co., Ltd., Beijing, China) according to the manufacturer's protocol. The extracted DNA samples were quantified with NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Massachusetts, USA). Next, they were frozen at -20 0C and kept until they were used for RAPD amplification by PCR. Five decamer primers (OPC-06: 5'-GAACGGACTC-3', OPR-12: 5'-ACAGGTGCGT-3', OPZ-03: 5'-CAGCACCGCA-3', OPZ-08: 5'-GGGTGGGTAA-3', OPZ-13: 5'-GACTAAGCCC-3'; Sigma-Aldrich Co., Tokyo, Japan) were dissolved in nuclease free water according to the technical data sheet. They were used for RAPD

amplification as described by [2]. Briefly, the PCR amplification reaction was carried out in a 25 µl reaction volume containing 22 µl PCR Master Mix (12.5 µl AmpliTaq Gold® 360 DNA Polymerase, 1 µl 360 GC Enhancer, 8.5 µl nuclease free water), 1 µl primer (working solution 15 µM), and 2 µl of DNA template (concentration approximately 50 ng/µl). The conventional PCR was performed in a controlled thermal cycler (TC9610; MultiGene™ OptiMax Thermal Cycler, New Jersey, USA).

The first cycle consisted of activation of PCR Master Mix at 95^0C for 10 min and denaturation of DNA template at 95 ^{0}C for 1 min, followed by primer annealing at 37 ^{0}C for 1 min and primer extension at 72 ^{0}C for 2 min. In the next 44 cycles, the period of denaturation, annealing and extension time remained as in the first cycle. The last step was primer final extension at 72 ^{0}C for 5 min and final hold at 4 ^{0}C. PCR products were separated by electrophoresis (Mupid®-exu; Mupid Co., Ltd., Tokyo, Japan) at 100 volts for 50 min on a 1.75 % agarose gel containing SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Invitrogen™, USA) using 1 x TAE buffer. The sizes of the amplified fragments were determined by using GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Thermo Scientific™, USA). DNA fragments were visualized and photographed using gel documentation system (Uvidoc HD6; UVItec Ltd., Cambridge, UK). The interpretation of band patterns were coded binarily by the visual scores [22]. Jaccard's coefficient of similarity was used for grouping of the parental and F1 progenies by unweighted pair-group method for arithmetic average analysis (UPGMA) cluster method. Dendrogram was drawn using cluster analysis options as available in MVSP 3.1 software.

3. Results and Discussion

In the present study, we analysed 8 yardlong bean genotypes consisted of three parental and 5 F1 progenies. RAPD-PCR revealed the present of amplicons and the diversity was observable based on the number of bands (Figure 1). In the analysis of samples, five selected primers generated 16 bands (loci) ranging in size from 280 to 1160 bp (Table 2), with an average number of bands per primer of 3.20. It is lower than a number of bands used in [2] and [23]. Primer OPC-06 produced 6 bands, primer OPZ-13 produced 4 bands, while primers OPR-12, OPZ-03, and OPZ-08 produced 2 bands, respectively. Four new bands, that were absent in the parental, were detected in the progenies. Some of them occured in only one progeny, like band 520 bp OPC-06 which was specific for the progeny FG x SP; band 310 bp OPC-06 was specific for the progeny AH x SP and band 280 bp OPC-06 was specific for the progeny SP x FG. The other band was detected in three progenies: band 460 bp OPZ-13 was present in SP x FG, FG x AH and SP x AH. This might indicate the presence of specific loci in the genotypes studied. Furthermore, detection of the band 500 bp generated by primer OPZ-08 in the parentals SP and AH, that were absent in the progenies, indicates that primer have potential to be used in identification of yardlong bean breeding materials.

Since variation occurs in many different types of mutational events, as well as in the annealing site of the primer and between two adjacent sites which responsible for the amplification [24], presence or absence of RAPD bands indicate the genetic changes in the hybrids genome, through the loss or rearrangement of its nucleotides [25]. It might affect the number and types of the amplified DNA bands (Table 3). Five RAPD primers scored 15 polymorphic bands out of 16 bands, while primer OPR-12 revealed a monomorphic band. The common or monomorphic bands among the yardlong bean parentals and their F1 progenies likely represent highly conserved regions in the genome. All primers revealed discriminating patterns which the number of bands ranged from 2 to 6. However, it indicates the presence genetic polymorphism in these genotypes which may be used in yardlong bean breeding. On the contrary, there were some unique bands only in three progenies generated by primer OPC-06, indicates the presence of specific loci in the genotypes studied. Although not all genotypes gave unique bands with every primer, certain primer, i.e. OPC-06, revealed more polymorphism than others. The present study revealed the average polymorphism of 90 % indicating the presence of greater genetic difference among the genotypes. Morover, the RAPD was found suitable for use with yardlong bean because of its ablity to generate consistent polymorphic markers. Therefore, the primers have used can be chosen to conduct a test of marker segregation in F1 progenies.

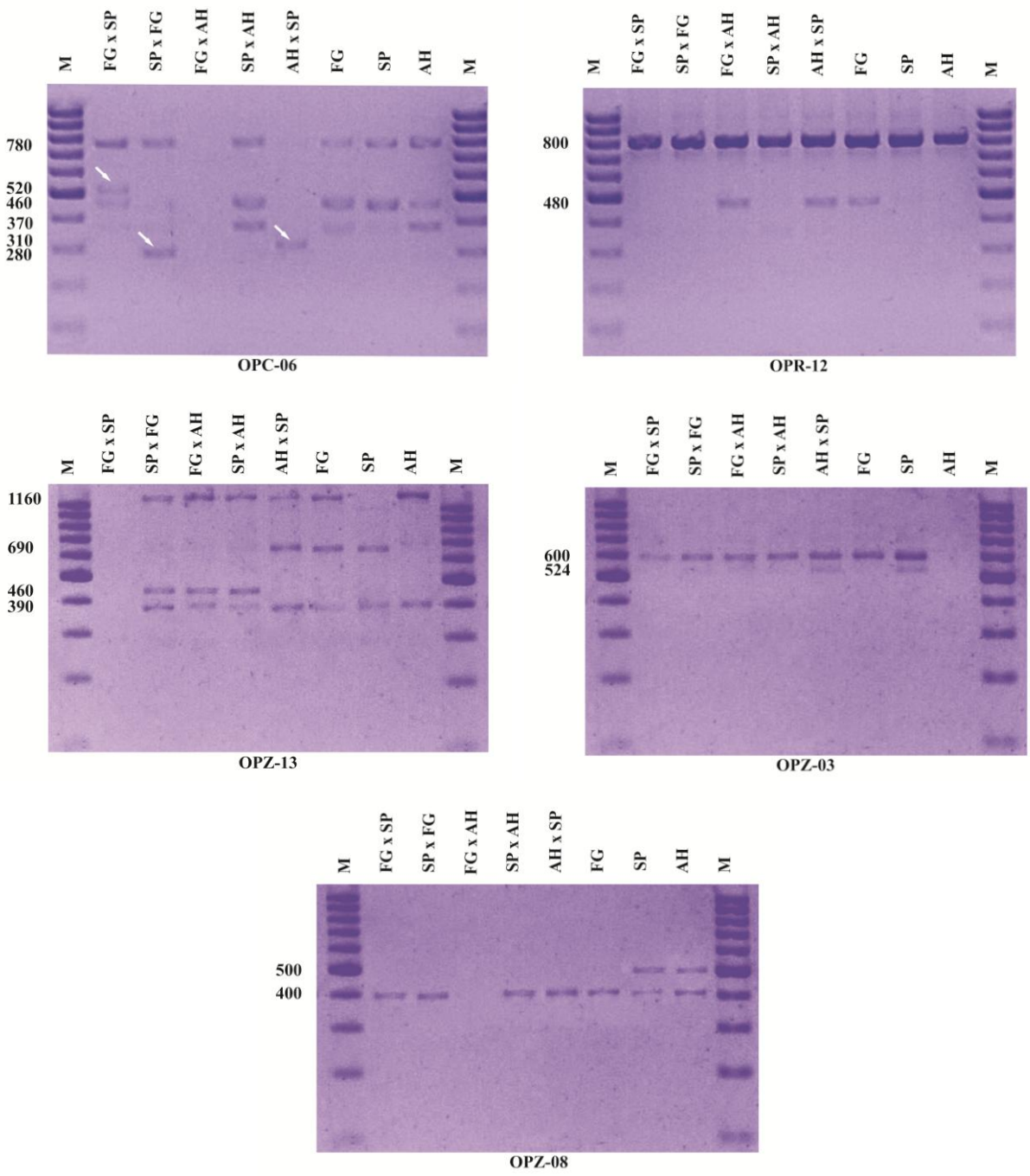

Figure 1. Amplification profiles of yardlong bean parentals and their F1 progenies DNA samples using RAPD markers. M = 1000 bp DNA ladder. White arrows indicate unique bands.

A genetic distance was performed by calculating the Jaccard's coefficient similarity. The distance ranged from 0.40 to 0.73, indicating that there was a high amount of genetic diversity among the genotypes. UPGMA dendrogram was drawn to vizualize relationships among yardlong bean parentals and their F1 progenies (Figure 2). The genetic relationship with coefficient that was more than 0.60 showed the close relationship among the genotypes [26]. Two clusters were formed, the largest cluster consisted of 5 genotypes including parentals and hybrids, while the second cluster contained 3 genotypes including only hybrids. They were separated in different clusters in the coefficient of 0.40.

The first cluster was separated on the coefficient of 0.45 into cluster A that includes FG x SP, and cluster B. Cluster B was separated on the coefficient of 0.63 into cluster i and ii. Cluster i was separated on the coefficient of 0.67 into cluster a and b. Interestingly, SP x AH (cluster a) is closer to FG (cluster a) than AH (cluster b) or SP (cluster ii). It explains that hybridization fall outside the range of parental variation but might have advantage of transient hybrid vigor, move desirable variation, and generate novel characters, however it shoud be assessed in the F2 generation and later to distinct it from heterosis or transgressive segregation [27].

Table 2. Bands appearance generated by five RAPD primers in yardlong bean parentals and their F1 progenies

Primer	Marker (bp)	Genotypes							
		FG x SP	SP x FG	FG xAH	SP x AH	AH x SP	FG	SP	AH
OPC - 06	780	+	+		+		+	+	+
	520	+							
	460	+			+		+	+	+
	370				+		+	+	+
	310					+			
	280		+						
OPR- 12	800	+	+	+	+	+	+	+	+
	480			+		+	+		
OPZ-03	600	+	+	+	+	+	+	+	
	524					+		+	
OPZ-08	500							+	+
	400	+	+		+	+	+	+	+
OPZ-13	1160		+	+	+	+	+		+
	690					+	+	+	
	460		+	+	+				
	390		+	+	+	+	+	+	+

Table 3. Number and types of the amplified DNA bands as well as the percentage of the total polymorphism generated by five RAPD primers in yardlong bean parentals and their F1 progenies

Primer	Monomorphic band	Polymorphic bands		Total band	Polymorphism (%)
		Unique	Shared		
OPC-06	0	3	3	6	100
OPR-12	1	0	1	2	50
OPZ-13	0	0	4	4	100
OPZ-03	0	0	2	2	100
OPZ-08	0	0	2	2	100

Reciprocal hybrids from crosses FG x SP (cluster A) and SP x FG (cluster iii) or SP x AH (cluster a) and AH x SP (cluster E), both were separated with its reciprocal on the coefficent of 0.40. Reciprocal differences are caused by unequal contribution of cytoplasmic determinants from male and female gametes to the zygote [28]. Possible explanations are nuclear-cytoplasmic interactions or parent-of-origin effects, which the differential transmisson of the organelles allows contrasting chloroplast, and mitocondrial combinatios, and identical nuclear genotypes [29]. When crosses and

their reciprocals are included, these estimated effects were not separated, showing the contribution of each parent to the cross combination when this particular parent is used as a male or female [30]. However, whether this substandard performance is attributable to the genotypes used as the paternal parents or due to the maternal parents genotypes is not evident from this study. So, it is important to point out that the genetic contribution of the parentals to various components of the yardlong bean F1 progenies are anequal in future studies. Moreover, this results of this study shows that RAPD can also be very efficient in evaluating the genetic relationships between closely related genetic materials, *i.e.* parentals and their F1 progenies.

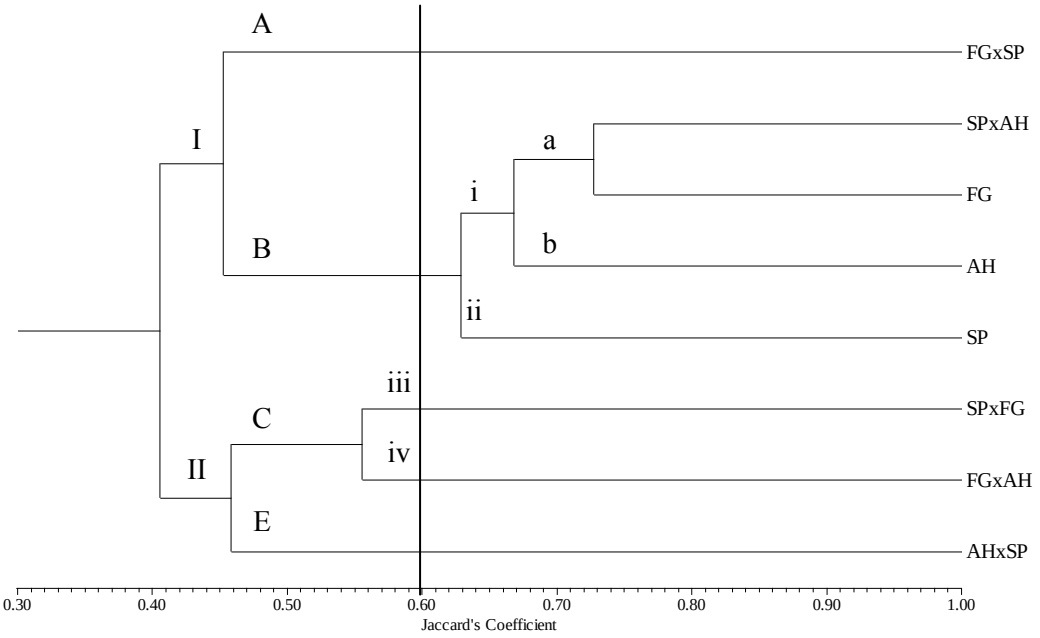

Figure 2. Dendrogram of yardlong bean parentals and their F1 progenies based on RAPD markers

4. Conclusions

Five primers generated 16 bands, ranging in size from 280 to 1160 bp, with the average number of bands per primer of 3.20. Cluster analyses using UPGMA procedure were performed using MVSP 3.1 software and produced a dendrogram with two clusters. Jaccard similarity matrices among yardlong bean parental and their F1 progenies varied between 0.40 – 0.73. The results indicate that use of RAPD markers on DNA samples can be fast way for differentiation of yardlong bean parental and their F1 progenies, as well as for evaluation of their genetic relationships.

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